

**Maize H3C4 promoter combined with the first intron of  
rice actin, chimeric gene comprising it  
and transformed plant**

The present invention relates to a new 5'  
5 regulatory sequence allowing the expression, in  
monocotyledonous plants, of a sequence heterologous to  
the said regulatory sequence, encoding a protein of  
interest. The present invention also relates to a  
chimeric gene comprising the said regulatory sequence,  
10 a heterologous sequence encoding a protein of interest  
and a 3' regulatory sequence allowing the expression of  
the protein of interest in a plant cell from a  
monocotyledonous plant, as well as a transformed  
monocotyledonous plant comprising the said chimeric  
15 gene and the means necessary for the transformation of  
plant cells and of plants.

Various promoters allowing the expression of  
sequences encoding proteins of interest in plants are  
known, are described in the literature, and have  
20 already allowed the development, to a commercial stage,  
of plants modified by genetic engineering. They are  
promoter sequences of genes which are expressed  
naturally in plants, in particular promoters of  
bacterial, viral or plant origin such as, for example,  
25 that of a gene for the ribulose biphosphate  
carboxylase/oxygenase small subunit (US 4,962,028) or  
of a gene of a plant virus such as, for example, that

of cauliflower mosaic (US 5,352,605). Promoters allowing the expression of heterologous genes in plants are in particular described in the following patents and patent applications: US 5,086,169, EP 0 353 908, 5 US 5,139,954, US 5,378,619, US 5,563,328, US 5,589,583, US 5,633,363, US 5,633,439, US 5,633,440, US 5,633,447, US 5,635,618, US 5,639,948 and US 5,639,952.

However, some of these promoters, and more particularly the promoters of plant origin, are not 10 functional in monocotyledonous plants.

*Arabidopsis* sp. histone promoters described in patent application EP 0,507,698 are for example known which are particularly efficient for allowing the expression of a heterologous gene in dicotyledonous 15 plants such as tobacco, oil seed rape or soya bean, which are not functional in monocotyledonous plants such as maize.

The rice actin promoter is a promoter known to allow the expression of heterologous genes in 20 monocotyledonous plants (US 5,641,876). However, the problem of identifying new functional 5' regulatory sequences for the expression of heterologous sequences in monocotyledonous plants still remains.

The present invention relates to a new DNA 25 sequence, a 5' regulatory element allowing the expression of a heterologous gene in a plant cell from a monocotyledonous plant, the said DNA sequence comprising, in the direction of transcription, a first

DNA sequence, which is a functional fragment of the sequence of the maize H3C4 promoter, and a second DNA sequence, which is a functional fragment of the sequence of the first intron of rice actin.

5           The sequence of the maize H3C4 promoter is in particular described by Brignon *et al.* (Plant. Mol. Biol., 22: 1007-1015, 1993). It is the *AluI* fragment of the maize H3C4 promoter, of about 1 kb, corresponding to bases -7 to -1029 relative to the ATG of the  
10       sequence encoding the maize histone H3C4.

          The sequence of the first intron of rice actin is in particular described in patent US 5,641,876.

          Functional fragment is understood according  
15       to the invention to mean any DNA sequence derived from the sequence of the maize H3C4 promoter or from the sequence of the first intron of rice actin, which reproduces the function of the sequence from which it is derived.

20           According to one embodiment of the invention, the functional fragment of the sequence of the maize H3C4 promoter comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO: 1) or a sequence homologous to the said sequence. Preferably,  
25       the functional fragment of the sequence of the maize H3C4 promoter consists of the DNA sequence described by the sequence identifier No. 1.

          According to one embodiment of the invention,

the functional fragment of the first intron of rice actin comprises the DNA sequence described by the sequence identifier No. 2 (SEQ ID NO: 2) or a sequence homologous to the said sequence. Preferably, the functional fragment of the first intron of rice actin consists of the DNA sequence described by the sequence identifier No. 2.

The DNA sequence, a 5' regulatory element, according to the invention may comprise, in addition, between the first and second DNA sequences, neutral DNA fragments which are generally necessary for the construction of the sequence according to the invention. These are DNA fragments comprising up to 30 base pairs, preferably up to 20 base pairs. Neutral DNA fragments are understood according to the invention to mean DNA fragments which do not substantially modify the respective functions of the first and second DNA sequences of the sequence according to the invention.

According to a preferred embodiment of the invention, the DNA sequence according to the invention comprises the DNA sequence represented by the sequence identifier No. 3 (SEQ ID NO: 3) or a sequence homologous to the said sequence. More preferably, the sequence according to the invention consists of the DNA sequence represented by the sequence identifier No. 3.

"Homologue" is understood according to the invention to mean a DNA sequence representing one or more sequence modifications relative to the reference

DNA sequence described by the sequence identifier No. 1, 2 or 3, and reproducing the function of the abovementioned sequences. These modifications may be obtained according to the customary mutation techniques, or alternatively by choosing the synthetic oligonucleotides which may be used in the preparation of the said sequence by hybridization. Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80%, more preferably at least 90%.

The present invention also relates to a chimeric gene (or an expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5' and 3' positions capable of functioning in plant cells from monocotyledonous plants, in which the 5' regulatory elements comprise the DNA sequence according to the invention defined above.

"Plant cell" is understood to mean according to the invention any cell derived from a monocotyledonous plant and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, monocotyledonous plant portions, monocotyledonous plants or seeds.

"Monocotyledonous plant" is understood according to the invention to mean any differentiated multicellular organism capable of photosynthesis, more particularly crop plants intended or otherwise as

animal feed or for human consumption, such as for example wheat, barley, oats, rice, maize, sorghum, sugar cane and the like.

According to the invention, it is also possible to use, in combination with the regulatory promoter sequence according to the invention, other regulatory sequences, which are situated between the promoter and the coding sequence, such as the sequences encoding transit peptides, either single, or double, and in this case optionally separated by an intermediate sequence, that is to say comprising, in the direction of transcription, a sequence encoding a transit peptide for a plant gene encoding a plastid localization enzyme, a portion of sequence of the mature N-terminal portion of a plant gene encoding a plastid localization enzyme, and then a sequence encoding a second transit peptide for a plant gene encoding a plastid localization enzyme consisting of a portion of sequence of the mature N-terminal portion of a plant gene encoding a plastid localization enzyme, as described in application EP 0,508,909. As transit peptide, there may also be mentioned the signal peptide for the tobacco PR-1a gene described by Cornelissen *et al.*

As regulatory terminator or polyadenylation sequence, there may be used any corresponding sequence of bacterial origin, such as for example the *Agrobacterium tumefaciens* nos terminator, or

alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633,317.

The coding sequence of the chimeric gene  
5 according to the invention may comprise any sequence encoding the protein of interest which it is desired to express in a plant cell or a monocotyledonous plant.

This may be a gene encoding a selectable marker such as a gene conferring on the transformed  
10 monocotyledonous plant new agronomic properties, or a gene for enhancing the agronomic quality of the transformed monocotyledonous plant.

Among the genes encoding selectable markers, there may be mentioned genes for resistance to  
15 antibiotics, genes for tolerance to herbicides (bialaphos, glyphosate or isoxazoles), genes encoding easily identifiable enzymes such as the enzyme GUS, genes encoding pigments or enzymes regulating the production of pigments in the transformed cells. Such  
20 selectable marker genes are in particular described in patent applications WO 91/02071 and WO 95/06128.

Among the genes conferring new agronomic properties on transformed monocotyledonous plants, there may be mentioned the genes conferring tolerance  
25 to certain herbicides, those conferring tolerance to certain insects, those conferring tolerance to certain diseases and the like. Such genes are in particular described in patent applications WO 91/02071 and

WO 95/06128.

As regulatory terminator or polyadenylation sequence, there may be used any corresponding sequence of bacterial origin, such as for example the

5 *Agrobacterium tumefaciens* nos terminator, or alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633317.

The present invention is particularly

10 appropriate for the expression of genes conferring tolerance to certain herbicides on transformed plant cells and on transformed monocotyledonous plants. Among the genes conferring tolerance to certain herbicides, there may be mentioned the *Bar* gene conferring

15 tolerance to bialaphos, the gene encoding an appropriate EPSPS conferring resistance to herbicides having EPSPS as target, such as glyphosate and its salts (US 4,535,060, US 4,769,061, US 5,094,945, US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783,

20 US 5,310,667, US 5,312,910, US 5,627,061, US 5,633,435, FR 2,736,926), the gene encoding glyphosate oxydoreductase (US 5,463,175), or alternatively a gene encoding an HPPD conferring tolerance to herbicides having HPPD as target, such as the isoxazoles, in

25 particular isoxafutole (FR 95 06800, FR 95 13570), the diketonitriles (EP 496 630, EP 496 631) or the triketones, in particular sulcotrione (EP 625 505, EP 625 508, US 5,506,195). Such genes encoding an HPPD



conferring tolerance to herbicides having HPPD as target are described in patent application WO 96/38567 and in unpublished patent application FR 97 14264, filed on 7 November 1997, whose content is incorporated  
5 herein by reference.

Among the genes encoding an appropriate EPSPS conferring resistance to herbicides having EPSPS as target, there may be mentioned more particularly the gene encoding a plant EPSPS, in particular from maize,  
10 having two mutations 102 and 106, which is described in patent application FR 2,736,926, called hereinafter double-mutant EPSPS, or alternatively the gene encoding an EPSPS isolated from *Agrobacterium* which is described by the sequences ID 2 and ID 3 of patent US 5,633,435,  
15 called hereinafter CP4.

Among the genes encoding an HPPD conferring tolerance to herbicides having HPPD as target, there may be mentioned more particularly the HPPD from *Pseudomonas* and that from *Arabidopsis*, which are  
20 described in patent application WO 96/38567.

In the case of the genes encoding EPSPS or HPPD, and more particularly for the above genes, the sequence encoding these enzymes is advantageously preceded by a sequence encoding a transit peptide, in  
25 particular the transit peptide called optimized transit peptide described in patents US 5,510,471 or US 5,633,448 whose content is incorporated herein by reference.

According to a preferred embodiment of the invention, the chimeric gene according to the invention comprises, in the direction of transcription, a 5' regulatory sequence according to the invention as  
5 defined above, functionally linked to a sequence encoding a fusion protein transit peptide/protein of interest, functionally linked to a 3' regulatory sequence, the different elements of the chimeric gene being defined above, the protein of interest being  
10 preferably an enzyme conferring tolerance to certain herbicides, more preferably enzymes of the EPSPS or HPPD type defined above.

Sequences encoding fusion proteins transit peptide/EPSPS, and more particularly OTP/double-mutant  
15 EPSPS are in particular described in patents US 4,940,835, US 5,633,448 and FR 2 736 926.

For the fusion protein OTP/CP4, persons skilled in the art will know how to construct the corresponding gene by taking the sequence encoding the  
20 CP4 described in patent US 5,633,435 and by following the procedure described in patents US 4,940,835, US 5,633,448 and FR 2,736,926 or in the examples below. The present invention also relates to a chimeric gene comprising, in the direction of transcription, an  
25 appropriate 5' regulatory sequence to ensure the expression of a heterologous gene in a plant cell, functionally linked to a sequence encoding a fusion protein OTP/CP4, functionally linked to a 3' regulatory

sequence. The 5' regulatory elements comprise not only the 5' regulatory elements according to the invention defined above, but also all the appropriate regulatory elements for allowing the expression of heterologous  
5 genes in plant cells from monocotyledonous or dicotyledonous plants which are known to a person skilled in the art or of the future, and in particular those described above.

The sequences encoding fusion proteins  
10 transit peptide/HPPD are described in patent application WO 96/38567.

The present invention also relates to a cloning or expression vector for the transformation of a plant cell or of a monocotyledonous plant, the  
15 transformed plant cells and plants containing at least one chimeric gene as defined above. The vector according to the invention comprises, in addition to the above chimeric gene, at least one replication origin. This vector may consist of a plasmid, a cosmid,  
20 a bacteriophage or a virus, which are transformed by introducing the chimeric gene according to the invention. Such vectors for transforming plant cells and monocotyledonous plants are well known to a person skilled in the art and are widely described in the  
25 literature. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a method

of transforming plant cells by integrating at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any appropriate known means with the vector according to the invention.

A series of methods consists in bombarding cells or cellular tissues with particles to which DNA sequences are attached. Another series of methods consists in using, as means of transfer into the plant, a chimeric gene inserted into an *Agrobacterium tumefaciens* Ti plasmid or an *Agrobacterium rhizogenes* Ri plasmid. Other methods may be used, such as microinjection or electroporation, or alternatively direct precipitation by means of PEG.

Persons skilled in the art will choose the appropriate method according to the nature of the plant cell or of the plant.

The subject of the present invention is also the plant cells or plants transformed and which contain at least one chimeric gene according to the invention defined above.

The subject of the present invention is also the plants containing transformed cells, in particular the plants regenerated from transformed cells. The regeneration is obtained by any appropriate method which depends on the nature of the species.

For the methods of transforming plant cells and of regenerating monocotyledonous plants, there may

be mentioned in particular Gordon-Kamm, W.J. et al.  
(*Transformation of Maize Cells and Regeneration of  
Fertile Transgenic Plants*, The Plant Cell, vol. 2, 603-  
618, July 1990), whose content is incorporated herein  
5 by reference, and the following patents and patent  
applications: US 5,177,010, US 5,187,073, EP 267,159,  
EP 604 662, EP 672 752, US 4,945,050, US 5,036,006,  
US 5,100,792, US 5,371,014, US 5,478,744, US 5,484,956,  
US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520,  
10 US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174,  
EP 486 233, EP 486 234, EP 539 563, EP 674 725,  
WO 91/02071 and WO 95/06128.

The present invention also relates to the  
transformed plants derived from the culture and/or the  
15 crossing of the above regenerated plants, as well as  
the seeds of transformed plants.

In the case where the chimeric gene according  
to the invention comprises a sequence encoding an  
enzyme conferring tolerance to a particular herbicide,  
20 the present invention also relates to a method of  
controlling weed in an area of a field comprising seeds  
or plants transformed with the said chimeric gene  
according to the invention, which method consists in  
applying to the said area of the field a dose of the  
25 said particular herbicide which is toxic to the said  
weed, without, however, substantially affecting the  
seeds or plants transformed with the said chimeric gene  
according to the invention comprising the said sequence

encoding an enzyme conferring tolerance to the said particular herbicide.

The present invention also relates to a method of culturing the plants transformed according to the invention with a chimeric gene according to the invention comprising a sequence encoding an enzyme conferring tolerance to a particular herbicide defined above, which method consists in planting the seeds of the said transformed plants in an area of a field which is appropriate for the culture of the said plants, in applying to the said area of the said field a dose of the said particular herbicide which is toxic to weeds should weeds be present, without substantially affecting the said seeds or the said transformed plants, and then in harvesting the cultivated plants when they reach the desired maturity and optionally in separating the seeds from the harvested plants.

In the above two methods, the application of the particular herbicide may be made according to the invention before sowing, before emergence and after emergence of the crop.

Advantageously, the enzyme for tolerance to a herbicide is an appropriate EPSPS, and in this case the herbicide is glyphosate or its salts, or the enzyme is an HPPD and the herbicide is chosen from the isoxazoles, in particular isoxafutole, the diketonitriles or the triketones, in particular sulcotrione.

The examples below make it possible to illustrate the invention without seeking to limit its scope.

1. Construction of a chimeric gene with a sequence  
5 encoding an HPPD:

The plasmids below are prepared so as to create an expression cassette comprising a maize H3C4 histone promoter combined with the untranslated 5' region of the first intron of the rice actin gene (ActI) described by Mc Elroy D. et al. (Plant Molecular Biology 15: 257-268 (1990)) directing the expression of the gene OTP-HPPD of *Pseudomonas fluorescens*.

pRPA-RD-195

15 The plasmid pRPA-RD-195 is a derivative of the plasmid pUC-19 which contains a modified multiple cloning site. The complementary oligonucleotides 1 and 2 below are hybridized at 65°C for 5 minutes, followed by a slow cooling down to 30°C over 30 minutes:

Oligo 4: 5' AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC  
GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG CATGC 3'

Oligo 5: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT  
GCATGCCTGC AGGTCGACTC TAGAGG 3'

20 The hybridized oligonucleotides are made double-stranded using the Klenow fragment of DNA polymerase I of *E. coli* to extend the 3' ends of each oligo using the standard conditions recommended by the manufacturer (New England Biolabs). The double-stranded oligo obtained is then linked in the plasmid pUC-19

previously digested with the restriction enzymes *EcoRI* and *HindIII* and made blunt-ended using the Klenow fragment of DNA polymerase I of *E. coli*. A cloning vector is thus obtained which comprises a multiple  
5 cloning site so as to facilitate the introduction of expression cassettes into a plasmid vector of *Agrobacterium tumefaciens* (Figure 1).

**pRPA-RD-2010:**

Insertion of the sequence "H4A748 promoter-  
10 OTP-double mutant EPSPS gene" of pRPA-RD-159 into the plasmid pRPA-RD-195.

The plasmid pRPA-RD-195 is digested with the restriction enzyme *SacI* and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs).  
15 The plasmid pRPA-RD-173 (described in patent FR 2,736,926) is digested with the restriction enzyme *SacI* and the DNA fragment containing the EPSPS gene is purified and linked into the plasmid pRPA-RD-195 prepared above. The clone obtained contains several  
20 unique restriction sites flanking the double-mutant EPSPS gene.

**pRPA-RD-1002**

Creation of an expression cassette OTP-HPPD for use in monocotyledonous plants. The plasmid pRP-P  
25 contains the optimized transit peptide (OTP) linked to the HPPD of *Pseudomonas fluorescens* followed by the polyadenylation site of nopaline synthase as described in patent application WO 96/38567. The components of



the plasmid pRP-P are the following:

- the optimized transit peptide (OTP) described in patents US 5,510,471 and US 5,633,448; this OTP consisting of 171 bp of the *Helianthus annuus* ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit transit peptide (Waksman G. et al. 1987. Nucleic Acids Res. 15: 7181) which are followed by the 66 bp of the mature portion of the *Zea mays* ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (Lebrun et al. 1987. Nucleic Acids Res. 15: 4360) which are themselves followed by the 150 bp of the *Zea mays* ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit transit peptide (Lebrun et al. 1987. Nucleic Acids Res. 15: 4360); the combination is therefore 387 bp;
- the coding region of the HPPD of *Pseudomonas fluorescens* described in patent application WO 96/38567; and
- the nopaline synthase (nos) terminator gene (polyadenylation zone of the nos gene isolated from pTi 37, 250 bp; Bevan M. et al. Nucleic Acids Res. 11: 369-385).

The plasmid pRP-P is digested with the restriction enzyme *BstEII*, treated with the Klenow fragment of DNA polymerase I of *E. coli* in order to make the fragment blunt-ended, and followed by digestion with the restriction enzyme *NcoI*. The DNA fragment obtained, containing the coding region OTP-

HPPD about 1.5 kb, is then purified. The plasmid pRPA-RD-2010 obtained above is digested with the restriction enzyme *BlpI*, treated with the Klenow fragment of DNA polymerase I of *E. coli* in order to obtain a blunt-ended fragment, and then digested with the restriction enzyme *NcoI*. The DNA fragment obtained, comprising the sequences of the plasmid vector, the H3C4 promoter combined with the untranslated 5' region and the first intron of the rice actin gene, is purified and the NOS polyadenylation site is purified. The two DNA fragments purified are linked so as to create an expression cassette OTP-HPPD comprising the maize H3C4 histone promoter (Brignon et al.) combined with the 5' untranslated region and the first intron of the rice actin gene (*ActI*) (*Act1* 5' UTR + intron 1) in order to control the expression of the coding region OTP-HPPD incorporating the NOS polyadenylation site (NOS polyA) (Figure 2).

## **2. Construction of a chimeric gene with a sequence encoding the double-mutant EPSPS:**

### **pRPA-RD-1010:**

Creation of an expression cassette OTP-double mutant EPSPS for use in monocotyledonous plants.

The plasmid pRPA-RD-109 contains the  $\beta$ -glucuronidase (GUS) gene of *E. coli* controlled by the maize H3C4 histone promoter (Brignon et al.) combined with the 5' untranslated region and the first intron of the rice actin gene (*ActI*) described by Mc Elroy D.

et al. (Plant Molecular Biology 15: 257-268, 1990). A diagram of this plasmid is represented in Figure 3. The plasmid pRPA-RD-109 is digested with the restriction enzymes *NcoI* and *EcoRI*, and the large DNA fragment (about 5 kb) containing the vector sequence, the GUS gene and the NOS polyadenylation site is purified. The plasmid pRPA-RD-2010 is digested with the restriction enzymes *NcoI* and *EcoRI*, and the DNA fragment (about 1.6 kb) containing the H3C4 promoter combined with the 5' untranslated region and the first intron of the rice actin gene (*ActI*) is purified. The two DNA fragments purified are linked in order to create an expression cassette OTP-double mutant EPSPS comprising the maize H3C4 histone promoter (Brignon et al.) combined with the 5' untranslated region and the first intron of the rice actin gene (*ActI*) in order to control the expression of the coding region OTP-double mutant EPSPS incorporating the NOS polyadenylation site.

### **3. Construction of a chimeric gene for tolerance to phosphinothricin (*bar* gene):**

The phosphinothricin acetyl transferase (PAT) encoded by the *bar* gene is an enzyme which inactivates a herbicide, phosphinothricin (PPT). PPT inhibits the synthesis of glutamine and causes a rapid accumulation of ammonia in the cells, leading to their death (Tachibana et al. 1986).

The plasmid used to introduce the tolerance to phosphinothricin as selection agent is obtained by

inserting the chimeric gene pDM 302 into the vector pSP72 of 2462 bp, marketed by Promega Corp.

(Genbank/DDBJ database accession number X65332) and containing the gene for resistance to ampicillin.

5           The plasmid pDM 302 of 4700 bp has been described by Cao, J., et al. Plant Cell Report 11: 586-591 (1992).

The various components of this plasmid are:

- the promoter of the rice actin gene described by  
10 Mc Elroy D. et al. Plant Molecular Biology 15: 257-268 (1990) consisting of 840 bp;
- the first exon of the rice actin gene consisting of 80 bp;
- the first intron of the rice actin gene consisting of  
15 450 bp;
- the region encoding the bar gene of 600 bp excised from the plasmid pIJ41404 described by White J. et al. Nuc. Acids Res. 18: 1862 (1990);
- the terminator of the nopaline synthase (nos) gene  
20 (polyadenylation zone of the nos gene isolated from pTi 37, 250 bp; (Bevan M. et al. Nucleic Acids Res. 11: 369-385).

#### 4. Transformation of maize cells:

25           The particle bombardment technique is used to introduce the genetic construct. The plasmids are purified on a Qiagen column and coprecipitated on M10 tungsten particles according to the Klein method (Nature 327: 70-73, 1987).

A mixture of metal particles, of the plasmid pRPA-RD-1002 and of the plasmid of Example 3 which are described above, is then bombarded onto embryogenic maize cells according to the protocol described by  
5 Gordon-Kamm, W.J. et al. (*Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants*, The Plant Cell, vol. 2, 603-618, July 1990).

**5. Regeneration and use of the bar gene as selection agent:**

10           The bombarded calli are selected on glufosinate until green sectors appear. The glufosinate-resistant positive calli are then converted to somatic embryos, and then placed under conditions which promote germination according to the operating  
15 conditions described by Gordon-Kamm, W.J. et al. (*Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants*, The Plant Cell, vol. 2, 603-618, July 1990). The young plants are transferred to a greenhouse for the production of seeds.

20   **6. Analysis of the progeny of the transformed plants:**

          The transformed plants obtained above are assumed in part to be transgenic, comprising a heterologous gene encoding OTP/HPPD conferring tolerance to isoxazoles such as isoxafutole. These  
25 transformed plants produced pollen, which fertilized ovules from a nontransgenic wild-type maize. The seeds obtained are selected on sand after treating with isoxaflutole.

The selection protocol is the following:

800 ml of Fontainebleau sand are placed in a tub of sides 15 x 20 cm. These tubs are then sprinkled with water and kept moist by supplying a nutrient solution consisting of 5 ml of Quinoligo (Quinoline) per litre of water. Twenty maize seeds are placed in the tubs, which are then treated with isoxaflutole by spraying at a rate of 100 g of active material per hectare (300  $\mu$ g of active material per tub). The tubs are then cultured in a greenhouse. The phytotoxicity is determined 14 [lacuna] after planting. According to the above conditions, the nontransformed plants exhibit 100% phytotoxicity whereas the transformed plants exhibit no phytotoxicity.

A comparative study was carried out with 20 maize lines transformed according to the invention and 20 maize lines transformed with a corresponding gene for which the sequence encoding the first intron of rice actin has been replaced with the sequence encoding the maize adh I intron. After treating by spraying very high doses of isoxafutole at a rate of 200 g of active material per hectare (600  $\mu$ g of active material per tub), the following results are obtained:

Rice actin intron according  
to the invention: 8/20 lines are tolerant  
Maize adh I intron: 3/20 lines are tolerant  
The results above demonstrate that the combination of the maize H3C4 promoter with the first

intron of rice actin according to the invention  
substantially enhances the expression of a protein of  
interest in transformed monocotyledonous plants  
compared with the combination of the same maize H3C4  
5 promoter with another intron of the state of the art.

## SEQUENCE LISTING

(iii) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO: 1:

5

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1021 base pairs  
 (B) TYPE: nucleotide  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(ix) FEATURE:  
 (A) NAME/KEY: maize H3C4 promoter  
 (B) LOCATION: 1..1021

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTTATGTGCA CCATTACTG TAATGCATAA TCATTTAATT GAATAGCAAA CTTTCTATT      60
ACTTCTTTAC TAACATAATT CTTGGTTTAA AAATTCAGTC CTCAACATTC ATTGCTCAAG     120
TATAAGTTGA GACTGTCAAA ATTACTATT TTATTCTTC ATATTTTTTT TCCTTATACA      180
CATTTTGGGC CTTACAATCC ATCATCTATA TCCATCCTTT CCGGTGTCCT CTAAAAGATT     240
CCATCCTCTG AATCTTATTC CTCTCCAATA ACGTTCTCTA AATCAGGTCT CTATAAGCAA     300
TACCTATATT AGAGACATTT TTTATTTTTT GTACATACAT ATTGTGCATA CTCTCAAATG     360
CATTATACAT ATTTAGTTTT ACTAAACCGA TTATTTAAAG TATTCAAACG GATGAAGAAC     420
TGTTTAGATA AATTCTATAT ATAGAGAATC CAGTAGCGTT CTCTAAATTT AGATGATTAT     480
TTAGAGGACG CTGTTAGAAA ACGTAAAAAA TTCTTTGATT ATTTATATTT AGGGTAGAGT     540
AGCCTTTATG CTTTATAGAT CTTTGGTGGA CCCAGCCTTA TACCGGTTAT TTTCGCGATT     600
GCGCCTCTCA TTTCACTCC AGCGCCCCAC ATTTTCACGT TTTCACCGAA GCGCCCAGCC     660
TGCTTAACCA ACAAAATTGT ACGGTGGCGC GGTTTTCAAA AGAAGTCGGA AACCATCTGC     720
ACCCACCGAC TAGTAGGCCC TCGGATCCTC CCTGATTAAG TCCTAGCCAA TAGGAGCCCA     780
GAACCAACCA TCACGCGGAT CGTCCCTACG CTTCCACCTC ATCGGCGCCG TCCATCTCCA     840
TCCAACACCT ATTCCGTAC CTTGCCCATC CTCCGAAAAA ATTCTCGGCT CGCGCTCCGC     900
ACCTACTACA AATACCCATC CCATCAGGAC GCATCGCATC ACTGCCAAAT CCCCCAGAAA     960
ATCAACACCT CCCAATTCCA CGCTGCCACC AACTCGCGGT CCTCCGCGCC AAGCACCAA     1020
G

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1021



## (2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 454 base pairs  
 (B) TYPE: nucleotide  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:  
 (A) NAME/KEY: intron 1 of rice actin  
 (B) LOCATION: 1..454
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

GTAACCAACC CGCCCTCTC CTCCTTCTTT CTCGGTTTTT TTTTCGTCT CGGTCTCGAT      60
CTTTGGCCTT GGTAGTTTGG GTGGGCGAGA GCGGCTTCGT CGCCAGATC GGTGCGCGGG      120
AGGGGCGGGA TCTCGCGGCT GGCCTCTCCG GCGGTGAGTC GGCCCGGATC CTCGCGGGGA      180
ATGGGGCTCT CGGATGTAGA TCTGATCCGC CGTTGTTGGG GGAGATGATG GGGCGTTTAA      240
AATTTGCGCA TGCTAAACAA GATCAGGAAG AGGGGAAAAG GGCACTATGG TTTATATTTT      300
TATATATTTT TGCTGCTGCT CGTCAGGCTT AGATGTGCTA GATCTTTCTT TCTTCTTTTT      360
GTGGGTAGAA TTTGAATCCC TCAGCATTGT TCATCGGTAG TTTTCTTTT CATGATTTGT      420
GACAAATGCA GCCTCGTGCG GAGCTTTTTT GTAG                                  454

```

## (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1565 base pairs  
 (B) TYPE: nucleotide  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:  
 (A) NAME/KEY: maize H3C4 promoter  
 (B) LOCATION: 27..1047
- (ix) FEATURE:  
 (A) NAME/KEY: intron 1 of rice actin  
 (B) LOCATION: 1102..1555
- (ix) FEATURE:  
 (A) NAME/KEY: synthetic cloning site  
 (B) LOCATION: 1..26

(ix) FEATURE:

(A) NAME/KEY: synthetic binding sequence

(B) LOCATION: 1048..1069

(xi) SEQUENCE DESCRIPTION: SEO ID NO: 3:

GAATTCCTCG	AGGTCGACGG	ATCCCCCTTA	TGTGCACCAT	TTACTGTAAT	GCATAATCAT	60
TTAATTGAAT	AGCAAACCTT	TCTATTACTT	CTTTACTAAC	ATAATCTCTG	GTTTTAAAAA	120
TCAGTCTCA	ACATTCATG	CTCAAGTATA	AGTTGAGACT	GTCAAAATTT	ACTATTTTTAT	180
TTCTTCATAT	TTTTTTTCT	TATACACATT	TTGGGCCTTA	CAATCCATCA	TCTATATCCA	240
TCCTTTCCGG	TGTCTCTAA	AAGATTCCAT	CCTCTGAATC	TTATTCCTCT	CCAATAACGT	300
TCTCTAAATC	AGGTCTCTAT	AAGCAATACC	TATATTAGAG	ACATTTTTTA	TTTTTTGTAC	360
ATACATATTT	GTCACTACTCT	CAAAATGCATT	ATACATATTT	AGTTTTACTA	AACCGATTAT	420
TTAAAGTATT	CAAACGGATG	AAGAAGCTGT	TAGA'AAATT	CTATATATAG	AGAATCCAGT	480
AGCGTTCCT	AAATTTAGAT	GATTATTTAG	AGGACGCTGT	TAGAAAACGT	AAAAAATCT	540
TTGATTATTT	ATATTTAGGG	TAGAGTAGCC	TTTATGCTTT	ATAGATCTTT	GGTGGACCCA	600
GCCTTATACC	GGTTATTTTC	GCGATTGCGC	CTCTCATTTT	CACTCCAGCG	CCCCACATTT	660
TCACGTTTTC	ACCGAAGCGC	CCAGCCTGCC	TAACCAACAA	ATTGGTACGG	TGGCGCGGTT	720
TTCAAAAGAA	GTCCGAAACC	ATCTGCACCC	ACCGACTAGT	AGGCCCTCGG	ATCCTCCCTG	780
ATTAAGTCCT	AGCCAATAGG	AGCCCAGAAC	CACCCATCAC	GCGGATCGTC	CCTACGCTTC	840
CACCTCATCG	GCGCCGTCCA	TCTCCATCCA	ACACCTATTC	CGTTACCTTG	CCCATCCTCC	900
GAAAAAATTC	TCGGCTCGCG	CTCCGCACCT	ACTACAAATA	CCCATCCCAT	CACGACGCAT	960
CGCATCACTG	CCAAATCCCC	CAGAAAATCA	ACACCTCCCA	ATTCCAGCT	GCCACCAACT	1020
CGCCGTCCTC	CGCGCCAAGC	ACCAAAGGAA	TTGGCCGCCA	CCGCGGTGGA	GCTCCTCCCC	1080
CCTCCCCCTC	CGCCGCCGCC	GGTAACCACC	CCGCCCTCT	CCTCTTTCTT	TCTCCGTTTT	1140
TTTTTTCGTC	TCGGTCTCGA	TCTTTGGCCT	TGGTAGTTTG	GGTGGGCGAG	AGCGGCTTCG	1200
TCGCCCAGAT	CGGTGCGCGG	GAGGGGCGGG	ATCTCGCGGC	TGGCGTCTCC	GGGCGTGAGT	1260
CGGCCCGGAT	CCTCGCGGGG	AATGGGGCTC	TCGGATGTAG	ATCTGATCCG	CCGTTGTTGG	1320
GGGAGATGAT	GGGGCGTTTT	AAATTCGCC	ATGCTAAACA	AGATCAGGAA	GAGGGGAAAA	1380
GGGCACTATG	GTTTATATTT	TTATATATTT	CTGCTGCTGC	TCGTCAGGCT	TAGATGTGCT	1440
AGATCTTCT	TTCTACTTTT	TGTGGGTAGA	ATTGGAATCC	CTCAGCATTG	TTCATCGGTA	1500

GTTCCTTTT TCATGATTG TGACAAATGC AGCCTCGTGC GGAGCTTTTT TGTAGGTAGA 1560

CC ATG

1565

## (2) INFORMATION FOR SEQ ID NO: 4:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 85 base pairs  
(B) TYPE: synthetic oligonucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC 60
CTCTAGAGTC GACCTGCAGG CATGC 85
```

## (2) INFORMATION FOR SEQ ID NO: 5:

- 10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 66 base pairs  
(B) TYPE: synthetic oligonucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC 60
TAGAGG 66
```